Hedgehog Signals Regulate The Proliferation Of Cardiac Progenitor Cells In Pharyngeal Arches

by

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A thesis submitted to Johns Hopkins University in conformity with the requirements for the degree of Master of Science

Baltimore, Maryland

May 2020

Abstract:

Congenital heart disease (CHD) is one of the most common live birth defects. The study of its underlying molecular and genetic mechanisms is crucial to our understanding of the origin of CHD and to the improvement of existing treatments. Previous studies have shown the importance of the Sonic Hedgehog (Shh) signaling pathway, among others, in the embryonic development of heart. Shh from distinct pulmonary and pharyngeal endoderm modulates second heart field (SHF) development and atrial septation. We provided evidence that the pharyngeal ectoderm may be another source of the Hedgehog signal. The conditional knockout of Shh and its paralog, Indian Hedgehog (Ihh), led to hypoplastic hearts missing the right ventricle and part of the outflow tract in embryonic day 9.5 mice. We used mouse embryonic stem cells (mESCs) to study how Hedgehog signaling controls the proliferation of SHF cardiac progenitor cells (CPCs), a potential contributing factor to the hypoplastic heart. By treating SHF CPCs with Hedgehog agonist and antagonist, we observed that Hedgehog activation increased proliferation within 24 hours after treatment while Hedgehog inhibition had the opposite effect. Furthermore, the heart phenotypes observed in Shh mutants are similar to those observed in mice that model Down syndrome (here, “Down syndrome mice). Disruption of Shh signaling in Down syndrome mice resulted in increased frequencies of complete atrioventricular septal defect (AVSD) in which the dorsal mesenchymal protrusion was absent. Further, we examined the interactions between Hedgehog signaling and trisomy in DS mice and identified two genes orthologous to chromosome 21, *Ripk4* and *Grik1*, that were misregulated beyond the expected 50% increase in expression level. In mESC, we found that *Grik1* overexpression was correlated with downregulation of *Ptch1*, a Hedgehog

pathway gene, at the cardiomyocyte stage. The activation of Hedgehog signaling suppressed *Grik1* transcriptional expression. The knockdown of Hedgehog pathway genes *Gli1* and *Gli2* resulted in normal the *Grik1* level back control level. This indicates a potential regulatory interaction between Hedgehog signaling and trisomy for *Grik1*.

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Dr. Roger H. Reeves, Ph.D.

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Introduction:

The primary function of heart is pumping oxygen- and nutrient-rich blood to maintain the functionalities of body. Its development requires precise spatial-temporal regulation to avoid structural and functional abnormalities. Congenital heart disease (CHD) is one of the most common developmental conditions [1], observed in 0.4% to 5% of live births [2]. The study of CHD and underlying molecular and genetic mechanisms is crucial to our understanding of the origin of CHD and to the improvement of existing treatments.

Previous studies have shown the importance of Hedgehog signaling [3], especially Shh, in the embryonic development of heart. Hedgehog signaling interacts with several cardiogenic transcriptional factors such as *Gata4* [4], *Tbx5* [5], *Foxf* [6,7], and *Bmp* [8] to modulate SHF development and atrial septation. Hedgehog signaling from distinct pulmonary and pharyngeal endoderm is required for inflow and outflow septation, respectively [9]. The Shh signaling is required within the dorsal mesocardium for its contribution to the atria; severe septal defects occur if Shh signaling is disrupted [10]. The loss of Shh also results in outflow tract (OFT) defects.

Mammals have three Hedgehog homologs, Sonic (Shh), Indian (Ihh) and Desert (Dhh), of which Shh is the best studied. Canonical Shh signaling is activated when the Shh molecule binds and inactivates the transmembrane receptor protein, Patched 1 (Ptch1), located on the target cell. Without the presence of the ligand Shh, Ptch1 suppresses the activity of another transmembrane protein, Smoothened (Smo). Ptch1 inhibition of Smo is abolished in response to Shh signaling. Then Ptch1 is internalized and degraded, which allows Smo to accumulate and activate downstream signaling cascade. This signaling cascade results in the translocation of Gli family proteins (Gli1,

Gli2 and Gli3) to the nucleus to initiate the transcription of target genes, such as Ptch1. The Gli family proteins are differentially regulated by Shh signaling [11]. Whereas Gli1 acts as an activator exclusively, Gli2 and Gli3 can switch between activator and repressor forms by proteolytic cleavage. Gli2 functions primarily as an activator and Gli3 predominantly acts as a repressor. Due to the complex processing nature of Gli2 and Gli3, Ptch1 and Gli1 are usually used as the direct indicators of Hedgehog signaling.

Our lab previously showed that the second pharyngeal arch (PA2) potentially provides a niche environment that controls the expansion of second heart field (SHF) cardiac progenitor cells (CPCs) that express the biomarker, Isl1 (Isl1+ CPCs) [26]. We examined the specific signaling pathway that governs the proliferation SHF CPCs in PA2 (data unpublished). Using transcriptional analysis and X-gal staining, we found Hedgehog signaling was highly active in PA2 and not in outflow tract (OFT) or the heart. The proliferation of mouse embryonic stem cell-derived CPCs (mESC-CPCs) that were cultured on PA2 feeder cells decreased significantly in responses to Hedgehog inhibition. Therefore, we hypothesized that Hedgehog signaling within PA2 regulates expansion of SHF CPCs.

In this study, we used both a mouse model and a newly developed mESC line to investigate whether our initial hypothesis is true. The mouse model contained an *Ap2-alpha-Cre* [12,13] driver that extensively labels the ectodermal region of PA2. The conditional knockout of both Shh and Ihh resulted in hypoplastic hearts on embryonic day 9.5. This result led us to plan experiments to determine whether the hypoplastic heart development was caused by defects of proliferation, migration, or differentiation (or a combination of these). [These experiments await reopening of the laboratory after the

COVID-19 pandemic]. For in vitro experiments, we used the mESCs reporter line, *Tbx1* RFP; *Hcn4* GFP to test the responses of SHF-derived cardiomyocytes to Hedgehog activation or inhibition. We observed that Hedgehog activation promoted the proliferation of SHF-derived cardiomyocytes and inhibition had the opposite effect.

Materials and Methods:

Mice Crosses and Genetics:

AP2-alpha^{Cre}; Shh$^{fl/fl}$; Ihh$^{fl/fl}$ mice were generated by crossing an *AP2-alpha^{Cre}; Shh$^{fl/+}$; Ihh$^{fl/+}$* male mouse with *Shh$^{fl/fl}$; Ihh$^{fl/fl}$* female mice. We previously linage traced AP2-alpha with ROSA-YFP, which labeled the pharyngeal ectoderm but not the foregut endoderm that has been reported to be the source of Hedgehog signaling. With the Cre-loxP system, we use the AP2-alpha driver to delete the expression of Shh and Ihh at the ectodermal region in second pharyngeal arch. Embryos were harvested at Embryonic Day 9.5 (E9.5). Theses mice were provided by Dr. Chulan Kwon of Johns Hopkins University, School of Medicine. For adult mice, 0.5 mm tail snips were obtained from 3-week-old mice. For embryos, yolk sacs were used instead. DNA was extracted by using Phire Animal Tissue Direct PCR Kit (ThermoFisher Scientific), followed by Polymerase Chain Reaction (PCR). Nuclease-free water, Promega GoTaq Green Master Mix, and primers designed by Dr. Brian Gibbs (supplied by Integrated DNA Technologies) were combined with the extracted DNA for PCR. The samples were then run on an Agarose gel (Shh, 2.5%; Ihh and Cre, 2.0%) with ethidium bromide for 30 minutes at 200 volts, which were imaged using ultraviolet imaging.

ES Cell Culture and Differentiation:

Embryonic stem cells (ESCs) line *Tbx1*-RFP; *Hcn4*-GFP was maintained in standard ESC culture 2i medium composed of GMEM, Leukemia Inducible Factor, Fetal Bovine Serum, Glutamax, Non-Essential Amino Acids, beta-Mercaptoethanol, and inhibitors PD and Chir. Cells were plated in a gelatinized 6-well plate or flasks and were

passaged until they reach 70%-80% confluency. Differentiation was initiated by switching from 2i media to Lif media, which are similar to 2i except that the extra Leukemia Inducible Factor and the two inhibitors were not included. Twenty-four hours later, Lif media were removed and washed cells were trypsinized for 3 minutes for dissociation. The dissociated cells were collected, centrifuged at 1100 rpm for 3 minutes, and resuspended in 10 mL of SFD +/+ media, which composed of IMDM, Ham's F12, N2 supplement, B27 supplement, 10% BSA in PBS, Glutamine, PenStrep, ascorbic acid, and 1-Thioglycerol. On Day 0, resuspended cells were seeded into AggreWell 400 (STEMCELL technologies), which was pre-coated with Anti-Adherence Rinsing Solution (STEMCELL technologies), to form embryoid bodies (EBs). After two-day culture, the EBs were transferred to culture plates with the treatment of Chir and Bmp4 on Day3 and Day 4 respectively. EBs were then replated in low-attachment plate or flask and sorted by SH800 sorter (Sony Biotechnology, Japan) on Day 5.5 or 6. Sorted RFP cells were plated in Nunc 384-well Clear Polystyrene Plates and treated with Smoothened Agonist (SAG, Tocris Bioscience), Cyclopamine (Selleckchem), and Recombinant Mouse Sonic Hedgehog (mShh, R&D Systems).

EdU Labeling, Immunochemistry, and Microscopy:

For EdU labeling, 10 mM EdU was injected intraperitoneally at 0.075 mg per gram bodyweight to pregnant mice at E9.0. Embryos were harvested immediately at 2 hours post injection. Upon collection, embryos were fixed in 4% paraformaldehyde in PBS for 40 minutes, and then transferred to 30% sucrose in PBS overnight. On the other day, embryo images were taken by an Axiocam microscope camera. Then they were

embedded in O.C.T. Compound (Fisher HealthCare) and flash frozen in a bath of 2-methyl-butanol with dry ice. The embryos were sectioned and stained using standard protocols. Antibodies used were goat Nkx2.5 (1:200; Santa Cruz Biotechnology) with Alexa 568 donkey anti-goat secondary antibody (1:500), mouse Troponin T (1:500; ThermoFisher Scientific) with Alexa 488 anti-mouse secondary antibody (1:500), rabbit Tbx5(1:250; Millipore Sigma) with Alexa 647 anti-rabbit secondary antibody (1:500), rabbit anti-phospho-Histone H3 (1:1000; Millipore Sigma) with Alexa 594 anti-mouse secondary antibody (1:500). Images were acquired with a Keyence BZ-X710 all-in-one microscope.

qPCR:

RLT Lysis buffer from QIAGEN was added to washed cells in cell culture plate. The RNA extraction and isolation followed the protocol of RNeasy Micro Kit from QIAGEN. The concentration of RNA was determined using a Nanodrop Spectrophotometer. Five hundred nanograms of produced cDNA using RT-PCR kit (ThermoFisher Scientific) of each sample were used for qPCR analysis.

siRNA, Constructs, and Transfection:

For *Gli1* and *Gli2* knockdown, mouse DsiRNA *Gli1* (mm.Ri.Gli1.13.1, Integrated DNA Technologies) and mouse DsiRNA *Gli2* (mm.Ri.Gli2.13.1, Integrated DNA Technologies) were used to transfect mESC tGFP cell line with Lipofectamine RNAiMAX kit (ThermoFisher Scientific). For *Ripk4* and *Grik1* overexpression, we used the plasmids designed and provided by Anna Moyer from Dr. Roger Reeves's lab and transfected

mESC tGFP cell line on differentiation day 4 with Lipofectamine 3000 (Life Technologies) in single cell suspensions.

Results:

Double knock-out of Shh and Ihh in PA2 leads to hypoplastic heart at E9.5

We previously linage-traced AP2-alpha+ cells with AP2-alpha-Cre; ROSA-YFP mice, which labeled the pharyngeal ectoderm (unpublished data not shown here) but not the foregut endoderm that has been reported to be the source of Hedgehog signaling earlier [9]. With the Cre-loxP system, we utilized the AP2-alpha driver to delete the expression of Shh and Ihh at the ectodermal region in PA2 to determine the effects of Hedgehog signals on the heart formation during the early stages. We crossed *Ap2-Cre; $Shh^{fl/+}$; $Ihh^{fl/+}$* with *$Shh^{fl/fl}$; $Ihh^{fl/fl}$* (Fig.1A) and harvested the embryos at Embryonic Day 9.5 (E9.5). Wildtype (WT) embryos, which were *Ap2-Cre; $Shh^{fl/+}$; $Ihh^{fl/+}$,* showed normal developing pharyngeal arches and heart (Fig.1B, 1B') with intact OFT, right ventricle (RV) and left ventricle (LV). No structural defects of heart or pharyngeal arches were observed in the E9.5 embryos of *Ap2-Cre; $Shh^{fl/fl}$; $Ihh^{fl/+}$* (Fig.1C, 1C'), single knockout of Shh. The pharyngeal arches and RV were absent in *Ap2-Cre; $Shh^{fl/fl}$; $Ihh^{fl/fl}$* double knockout (DKO) embryos (Fig.1D,1D'). The DKO embryos also displayed slight developmental deformation, such as smaller total embryo size. The embryos were cryo-sectioned and immunostained with *Nkx2.5*, a marker of SHF, cTnT, a marker of myocardial tissue, and Tbx5 that specifically expressed in the first heart field (FHF). The immunostaining results further confirmed that Shh/Ihh DKO causes single chamber formation with disappeared RV in E9.5 embryos (Fig.2A-C).

Deficiency of hedgehog activity diminishes proliferation of cardiac progenitor cells in the SHF

The next question to be answered is if the absence of RV and part of OT observed in Shh/Ihh DKO mouse was due to differential proliferation, apoptosis, or migration of SHF CPCs. To determine if proliferation is responsible for the loss of RV in the mutant, we plan to perform EdU pulse-tracing experiment [26]. The EdU is a proliferation assay that could be directly used to quantitate new synthesized DNA through detecting the fluorescent tag attached to deoxyribonucleoside analog. On E9.0, 10 mM EdU is going to be injected intraperitoneally at 0.075 mg per gram bodyweight to pregnant mice. After 2, 4, and 8 hours post-EdU injection, embryos are harvested for EdU detection followed by immunostaining with SHF progenitor marker Isl1, cardiac troponin (cTnT), cell mitosis marker phosphor-histone H3 (PH3), and DAPI. If the percentage of triple-positive (Isl1+, PH3+, EdU+) cells in Isl1+ cells within PA2 in the DKO mutant is significantly smaller than that of wild-type embryo at the same time point, the proliferation of CPC in PA2 diminished by the lack of Hedgehog signals.

To examine if migration is influenced by Hedgehog DKO, we plan to perform a dual injection experiment in which both EdU and BrdU are going to be sequentially injected at a 4-hr interval. The BrdU assay is another type of proliferation assay that requires DNA denaturation. Four hours post-BrdU injection or 8 hours post-EdU injection, embryos are harvested, fixed, and sectioned for EdU and BrdU detection. The sections are co-stained with Isl1 and DAPI. In wildtype, EdU+ progeny should proliferate and migrate out of PA2 (Isl1+ region) while BrdU+ progeny remains within PA2. If no migration of EdU+ progeny

observed and EdU+ region doesn't extend beyond the BrdU+ region in the DKO mutant, then the movement of CPC is restricted to PA2.

All the above speculative observations assume only proliferation or migration is affected at one time. If both of them are perturbed by the deficiency of Hedgehog signals, the observation will be much more complicated and further experiments may be required.

Hedgehog signaling affects the expansion of SHF CPCs-derived cardiomyocyte

To determine if Hh signaling plays a role in the expansion of SHF CPCs, mESCs were differentiated to CPCs (Fig.3A) and sorted for RFP+, Tbx1+cells (Fig.3A), i.e., SHF CPCs. Cells seeded on gelatin-coated plates were treated with Smoothened agonist (SAG, Hh activator) and Cyclopamine (Hh inhibitor) for 24 hours. The capability of SAG to activate the Hh signaling pathway was confirmed using qPCR to demonstrate increased transcription of *Gli1* and *Ptch1* after treatment (Fig.4A-B). Treatment with 125 μM SAG increased Gli1 expression by 51.5-fold and 250 μM of SAG increased it by 88.0 folds. The average numbers of cardiomyocytes (stretched, triangular-shaped cells) of 125 μM SAG and 250 μM of SAG counted in 10X fields (Fig.3B) were 163 and 183, respectively. As we expected, activation of Hedgehog signaling by SAG prompted the proliferation of RFP+ SHF cardiomyocytes compared to control (Fig.3C). The suppression of Hedgehog signaling by the Smo antagonist, Cyclopamine, was also verified as Gli1 expression decreased by 71% and 46% of that in control in responses to 1 μM and 10 μM treatments, respectively. That contradicts to our expectation that the extent of suppression is positively correlated to the dosage of Cyclopamine. The declines in cell number of 1 μM and 10 μM Cyclopamine treatment groups relative to control were not as

dramatic as that of group with treatment of both SAG and Cyclopmaine (Fig.3C). The possible explanation was that the re-plated cells on gelatin-coated surface lacked a source of Hedgehog molecules. The signaling was at baseline level and not fully activated causing changes were hard to be observed. It may also be caused by technical failures. All we can conclude from those preliminary results is that the activation of Hedgehog promotes the expansion of mESCs SHF-derived cardiomyocytes.

For future experiment, we need to optimize the dosage of Cyclopamine to see if the proliferation rate of mESCs SHF-derived cardiomyocytes is negatively correlated with its concentration as observed here. Except to cell count, we need also perform EdU staining as well as PH3 staining to examine how Hedgehog signals influence the proliferation of mESCs SHF-derived cardiomyocytes at cell cycle level. Since mESC-system is sensitive to time, we plan to culture mESCs SHF-derived cardiomyocytes in SAG/Cyclopamine-treated media longer to see if the cell responses vary with time.

Grik1 overexpression suppress Hedgehog signaling mRNA expression in SHF derived cardiomyocytes

Down syndrome (DS) is caused by inheritance of three copies of human chromosome 21. The prevalence of CHD in infants with DS is about 40%, which is over 133 times higher than in normal children [14], and AVSD is one of the typical malformations. The atrioventricular septum is formed by the fusion of atrial septum, dorsal mesenchymal protrusion (DMP), and endocardial cushion [15]. Recent evidence indicates that DMP malformation is the contributing factor to AVSD [16,17,18]. In mice with reduced Shh signaling, similar heart phenotypes were observed to those in Trisomy

mouse models [19,20,21]. Besides, DMP is hypoplastic or absent if Shh signaling is disrupted in DMP progenitors or in the SHF [9]. Shh signaling may play an important role in formation of the DMP as deletion of the Shh pathway activator, Smo, from the SHF resulted in compromised DMP formation and AVSD [22]. As an extension of this thesis project, we also examined how Shh signaling interacted with identified risk genes found in DS.

In our collaborative project with Dr. Roger Reeves's lab, we studied molecular and genetic mechanisms underlying high AVSD penetrance in DS. We performed RNA-sequencing analysis using cells collected from posterior SHF from E9.5 mouse embryos of *Isl-Cre; Dp16; Smo*$^{fl/fl,}$ a new mouse model with 100% AVSD we recently developed. Our unpublished data showed that both *Ripk4* and *Grik1* were among the most differentially expressed genes in Isl1+ SHF cells. This is in accordance with Dr. Antonarakis's study in which his group identified two CHD risk alleles on chromosome 21 in patients with Down Syndrome (DS): *Ripk4* and *Grik1* [23].

To mimic the high expression level of *Ripk4* and *Grik1* caused by trisomy21, we overexpressed both of them using transfecting tGFP cell line, a non-DS mESC cell line, with plasmids provided by Dr. Roger Reeves's lab. The GFP+ cells were FACS sorted and cultured on gelatin-coated plates for 24, 48 and 72 hours and then harvested for qPCR analysis (Fig. 5A). To examine the independent and combinational effects of *Ripk4* and *Grik1* on the Hedgehog signaling pathway, we designed 7 conditions: three control conditions including the baseline control, 100 nM SAG-treated control, and 100 nM SAG plus 10 μM Cyclopamine-treated control, *Ripk4* overexpression (OE), *Grik1* OE, *Ripk4*+*Grik1* OE and *Ripk4*+*Grik1* OE with Cyclopamine treatment (Table 1). The cells

were at cardiac progenitor stage 24 hours after treatment and entered the cardiomyocyte stage after 72 hours. Overexpression of *Ripk4* and *Grik1* was confirmed by qPCR (Fig.5 B-C). Within 24 hours, Hedgehog pathway genes *Ptch1*, *Gli1*, *Gli2* and *Gli3*, were not differentially expressed in any conditions compared to control in response to *Ripk4/Grik1* OE. However, *Grik1* overexpression induces significant downregulation of *Ptch1* and *Gli1*, whereas *Gli2* and *Gli3* (Fig.5D-G) were upregulated about 72 hours after treatment. In summary, the overexpression of *Grik1* induced downregulation of Hedgehog signaling pathway. This result indicated that *Grik1* may be an important regulator of Hedgehog signaling in individuals with DS, which is a potential mechanism that causes increased AVSD penetrance among DS patient compared to normal people.

Hedgehog activation causes downregulation of Grik1 and Gli2 knockdown recover Grik1 expression in SHF-derived cardiomyocytes

Next, we were interested to see if the *Ripk4/Grik1* and Hedgehog mutually regulate each other. To determine that, we used siRNA to knockdown *Gli1* and *Gli2*, major effectors of the canonical Hedgehog signaling cascade, and performed qPCR at 24, 48 and 72 hours after transfection to test the expression of *Ripk4* and *Grik1*.All reactions were treated with SAG except baseline control (Table 2) because the dissociated cells in plates didn't have a source of Hedgehog signals as they do in vivo. It would be hard to observe changes in expression if the Hedgehog pathway was not activated. Similar to the *Ripk4/Grik1* OE experiment, we didn't see any significant changes within 24 and 48 hours. When cells became cardiomyocytes (72 hours), interaction regulation of Hedgehog signaling on *Grik1* was observed. The knockdown of *Gli1* was successful as both *Gli1*

and *Ptch1*, two direct readouts of the Hedgehog pathway, were greatly suppressed (Fig.6 A and C). Although the *Gli2* expression level didn't reduce in response to either *Gli1* or *Gli2* KD (Fig.6B), the *Gli1* expression level went down (Fig.6A). No changes in the *Ripk4* level were observed in either *Gli1* or *Gli2* knockdown conditions. In responses to Hedgehog activation by SAG treatment, the *Grik*1 expression level was greatly reduced by 5.71 folds compared to baseline control (Fig.6D). In *Gli1* and *Gli2* KD, *Grik1* expression increased by 2.95 folds and 7.51 folds relative to SAG-treated control, respectively (Fig.6E). Knockdown of *Gli2* brought the *Grik1* level back to that of baseline control. Thus, manipulation of the Hedgehog signaling pathway is correlated with changes in expression of *Grik1* but not *Ripk4*. Combined with our observations in the *Grik1* OE experiment, it appears that *Grik1* and Hedgehog signaling interact in SHF derived cardiomyocytes.

Discussion:

Previous studies have shown that Shh ligands are produced from cells that reside in pharyngeal endoderm and pulmonary endoderm, which are necessary for arterial septation and outflow tract septation, respectively [24]. Our results here suggest that pharyngeal ectoderm may be another source of Hedgehog signaling. The conditional knockout of both Shh and Ihh within pharyngeal ectoderm using Ap2-alpha driver led to single-chamber hearts on E9.5 in mice. The overall size of mutant embryos was smaller than that of wildtype. The right ventricle and part of outflow tract, which are derived in part from SHF, were absent in double knockout embryos. Further, pharyngeal arches were malformed or absent. Thus, Hedgehog signals from pharyngeal ectoderm played important roles in the development of SHF-derived regions. Whether this defect was caused by insufficient proliferation, increased apoptosis, both of them and failure in cell migration. A next step to determine which of these mechanisms is involved would be to perform EdU pulse-tracing experiment for proliferation, dual injection of EdU and BrdU for migration and test Caspase expression for apoptosis in sagittal sections of E9.5 embryos.

To investigate how the Hedgehog signaling regulate SHF CPC proliferation and cell fate, we utilized an *in vitro* model to manipulate Hedgehog signaling. The proliferation rate of SHF CPC-derived cardiomyocytes increased in response to Hedgehog activation by SAG within 24 hours, as observed in Fig.3 B&F. The cell counts of cultures treated with 125 μM or 250 μM SAG group were 1.42-fold and 1.56-fold higher than that of control, respectively. However, the cell counts of Cyclopamine-treated cultures didn't show the presumed positive correlation with Cyclopamine dosage. A possible explanation was that

replated cells on gelatin-coated surface lacked a source of Hedgehog molecules. Thus, the signaling was at baseline level and not fully activated causing changes were hard to be observed. It could also be caused by technical failures. Due to the pandemic issue, we were not able to replicate the experiments. In results we did what suggested to us is that the protocol of Hedgehog inhibition need to be adjusted. All we can conclude from those preliminary results is that the stimulation of Hedgehog signaling pathway promotes expansion of mESCs SHF-derived cardiomyocytes within 24 hours after treatment. In future, we plan to optimize the concentration of SAG and Cyclopamine administrated to mESCs SHF-derived cardiomyocytes. To assess how mESCs SHF-derived cardiomyocytes react to stimulation and inhibition in the perspective of proliferation at cell cycle level, EdU and PH3 staining are required. We need more evidence to support our preliminary conclusion. For all above experiments, we didn't take account of cell-cell interactions between SHF CPCs and the interactions between SHF and FHF populations. Instead of isolating SHF cells on Day 6 via FACS sorting, we plan to keep the embryoid bodies intact, manipulate the Hedgehog signaling through SAG and Cyclopamine treatment, and test how SHF CPCs will response in the perspective of proliferation.

Hoffmann's studies [7, 9] showed that early in heart development, Hedgehog-responsive cells migrate from SHF and contribute to the primary atrial septum, DMP, endocardial cushions and pulmonary trunk. The malformation of DMP leads to complete AVSD with an incidence rate of 20% in patients with DS. Multiple lines of evidence implicate a Shh response deficit in trisomic cells as a contributor to multiple features of DS [25]. Therefore, we were interested to determine whether Hedgehog mitogens interact with genes on chromosome 21 in human, such as *RIPK4* and *GRIK1* [23]. In our qPCR

analysis, we found *Grik1* overexpression caused significant downregulation in *Ptch1* and *Gli1*, whereas *Gli2* and *Gli3* (Fig.5D-G) were upregulated about 72 hours after treatment. The activation of Hedgehog suppressed Grik1 expression at transcriptional level (Fig.6E) while inhibition of the Hedgehog pathway rescued Grik1 expression back to baseline (Fig.6E). Those preliminary results suggest a potential negative regulation loop between Grik1 and Hedgehog (Gli1 and Gli2) (Fig.6F). Further experiments are needed to validate this hypothesis. We can knockdown Grik1 in mESCs cell line and repeat the same analysis as we did for Grik1 overexpression.

In summary, our results provide evidence that the ectodermal region of the second pharyngeal arch in mouse is a potential source of Hedgehog signals that influence the development of SHF-derived regions, RV and OFT, probably through controlling the proliferation of SHF CPCs. And we reveal a possible regulatory interaction between Grik1 and Hedgehog pathway genes. It paves a path for our future studies of genetic and molecular mechanisms underlying AVSD development in DS.

Tables:

Table 1: Treatment conditions of *Ripk4/Grik1* OE experiment

Conditions	Details
Control	Empty
SAG	SAG(100 nM)
SAG + Cyc	SAG(100 nM) + Cyclopamine (10 uM)
Ripk4 OE	SAG(100 nM) + DNA 5 ug/uL
Grik OE	SAG(100 nM)+ DNA 5 ug/uL
Both OE	SAG(100 nM)+ DNA 5 ug/uL (Ripk4 & Grik1)
Both OE + Cyc	SAG(100 nM)+ Cyclopamine (10 uM) + DNA 5 ug/uL (Ripk4 & Grik1)

Table 2: Treatment conditions of Gli1/Gli2 KD experiment

Conditions	Details
Control	Empty
SAG	SAG(100 nM)
Gli1/Gli2/Gli3 KD	siRNA 1pmol + SAG(100 nM)

Figures:

A

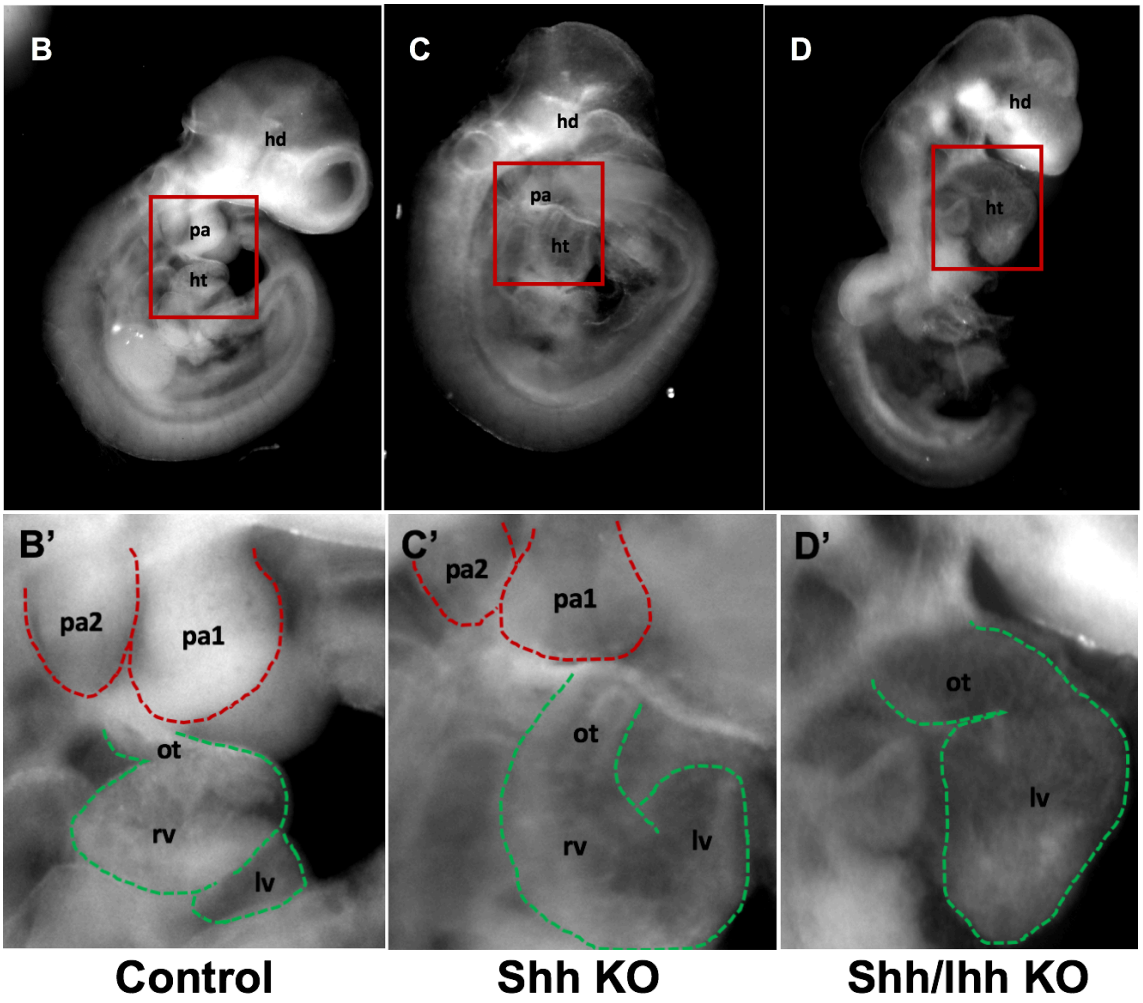

Figure 1: Double knock-out of Shh and Ihh in PA2 leads to hypoplastic heart at E9.5 (A) Scheme for mating strategy to generate Shh and Ihh double knockout embryos. (B-D) Lateral views of control: *Ap2-Cre; Shh$^{fl/+}$; Ihh$^{fl/+}$* (B), *Ap2-Cre; Shh$^{fl/fl}$; Ihh$^{fl/+}$* (Shh KO, C), *Ap2-Cre; Shh$^{fl/fl}$; Ihh$^{fl/fl}$* (Shh/Ihh DKO, D) embryos. (B'-D') Enlargement of boxed arears in (B-D), showing normal, normal, hypoplastic PA2 and heart in control (B'), Shh KO (C'), and Shh/Ihh DKO (D'), respectively.

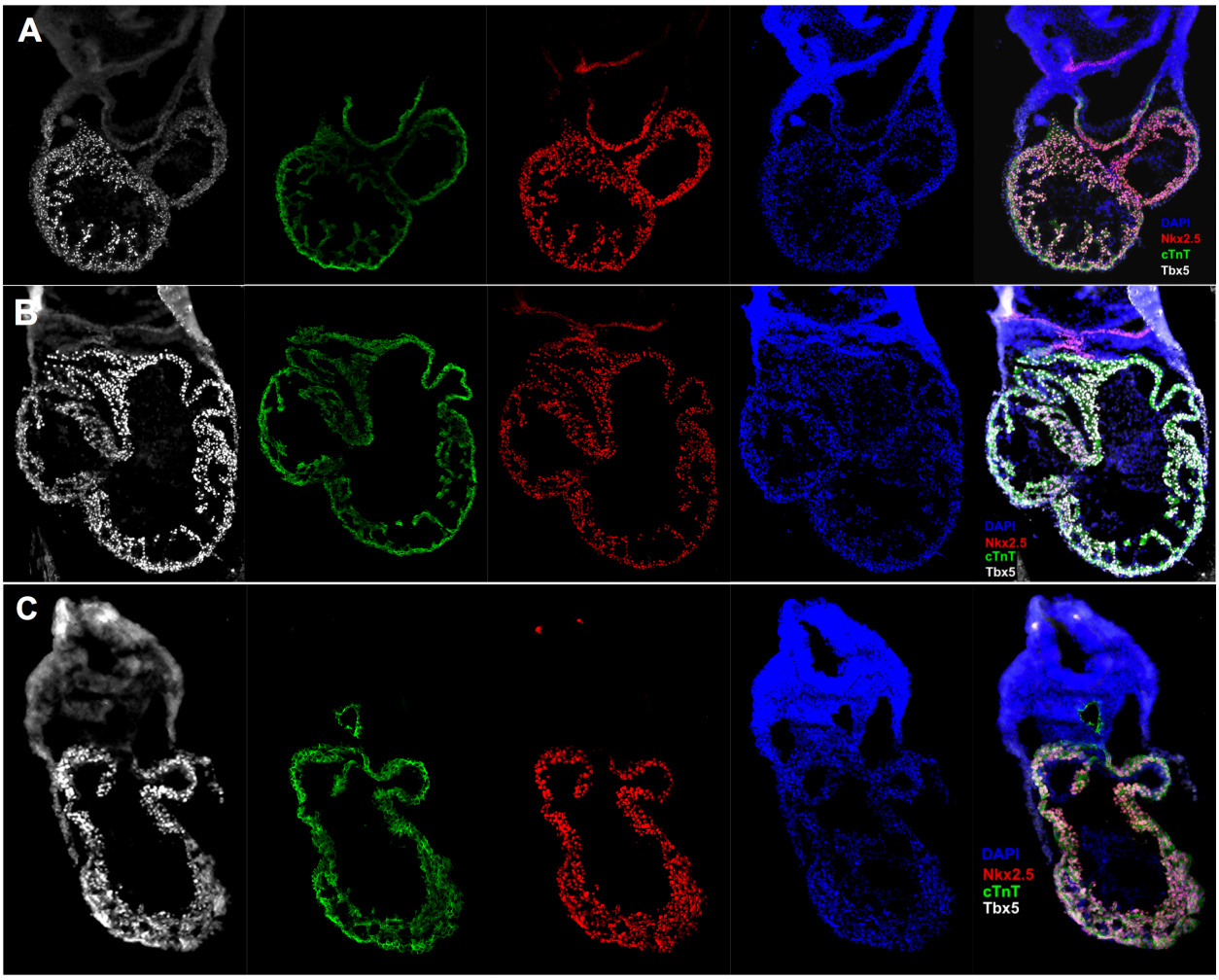

Figure 2: Immunostaining of E9.5 mouse embryos with double knock-out of Shh and Ihh in PA2.
(A-C) Cross-sections of control (A), Shh KO (B), and Shh/Ihh DKO (C). Sections stained for Nkx2.5 (red), cTnT (green), Tbx5 (white), and DAPI (blue). Pharyngeal arches (red) and outflow tract/right ventricle (green) are outlined in dashes. Hd, head; pa, pharyngeal arch; ht, heart; ot, outflow tract; rv, right ventricle; lv, left ventricle.

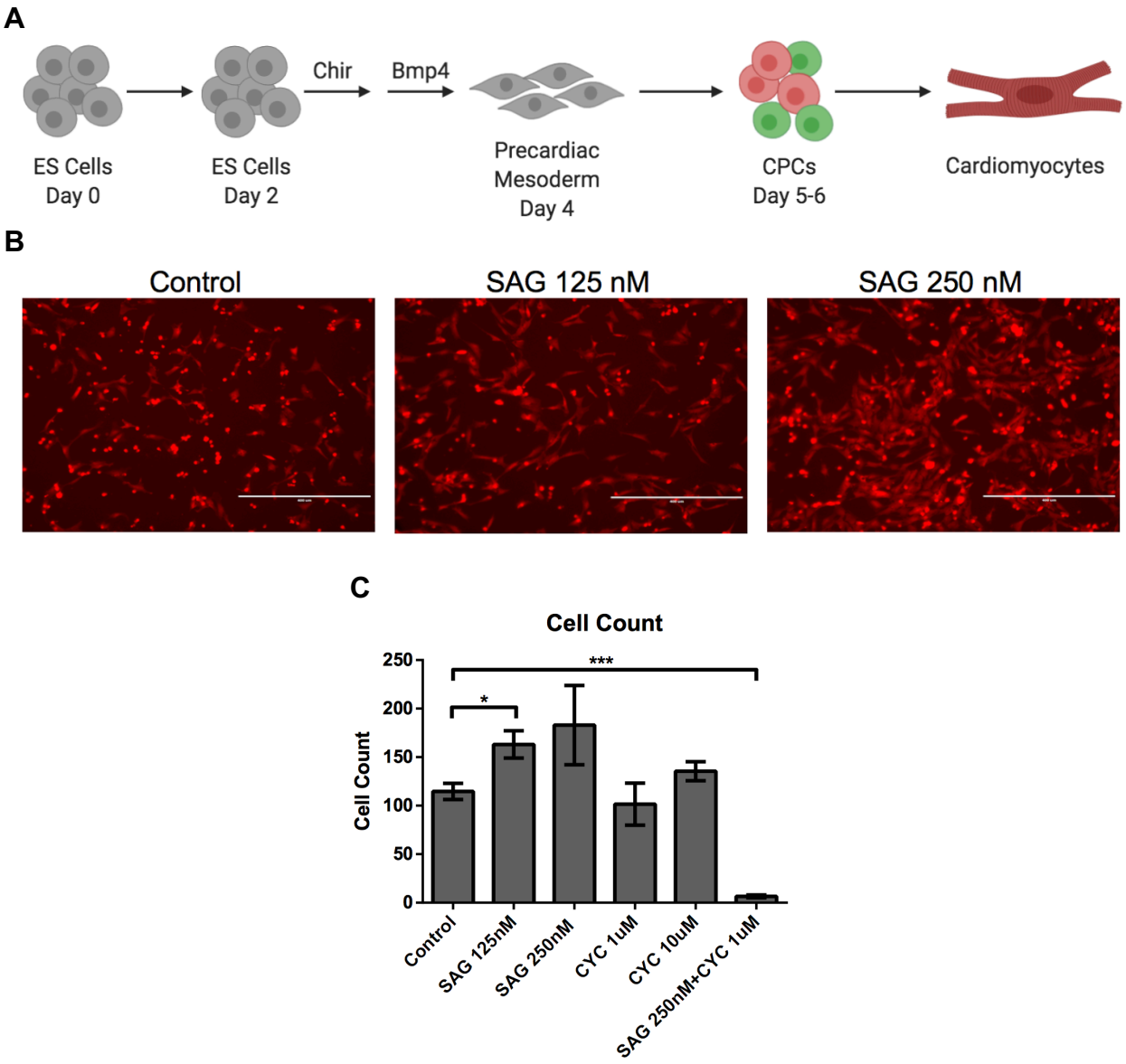

Figure 3: Hedgehog signaling affects the expansion of SHF CPCs-derived cardiomyocyte *in vitro*.
(A) Workflow of mESCs differentiation. (B) Images of RFP+ SHF cardiac progenitor cells 24 hours after replating and treatment with SAG, scale bar = 400 μm. Stretched and triangular-like cells were cardiomyocytes. (C) Cell count per view square as shown in (B). SAG, Smoothend against, CYC, Cyclopamine.

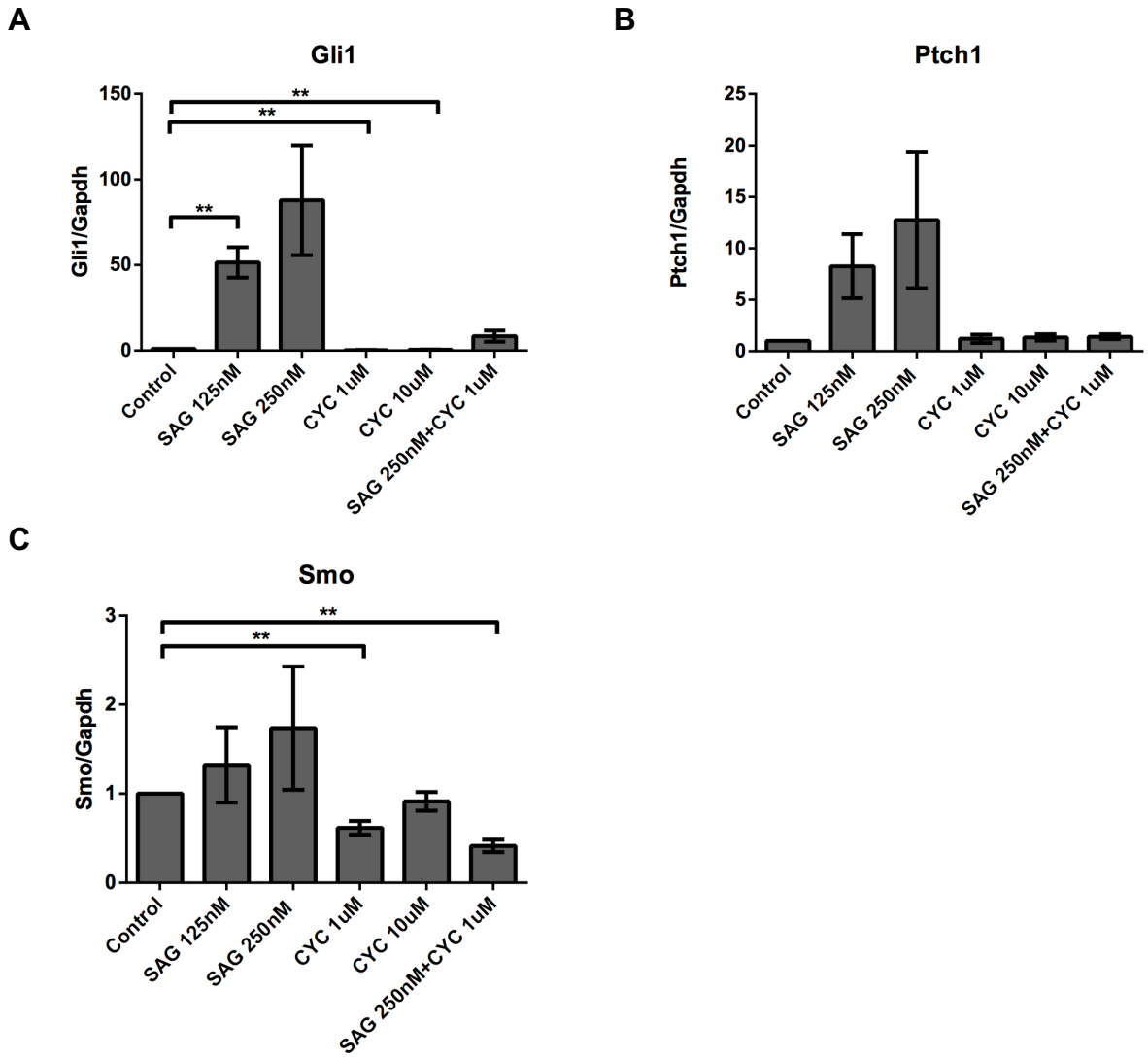

Figure 4: Expression level of Hedgehog signaling pathway genes under different treatment conditions monitored by qPCR.

(A-C) The transcriptional expression analysis of (A) *Gli1*, (B) *Ptch1*, and (C) *Smo* were examined by qPCR. Samples were normalized by *Gapdh* expression and the control was set as 1.0. For each condition, n = 3, paired t-test. Error bars indicate standard error of mean. Asterisks indicate statistical significance at P≤0.05.

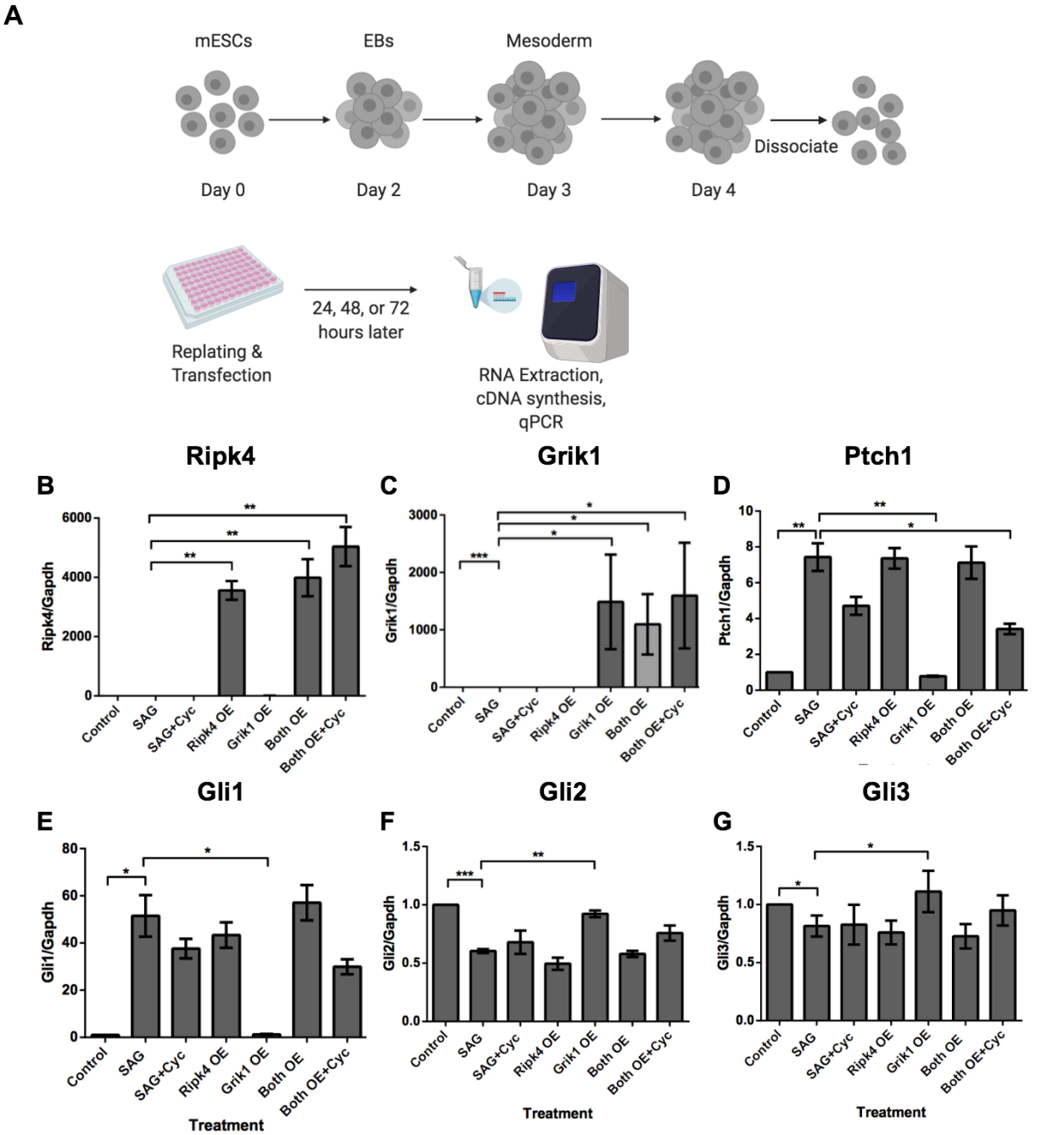

Figure 5: Interactions between mutant genes (*Ripk4* and *Grik1*) associated with high risk of AVSD in DS and Hedgehog signaling pathway.

(A) Workflow of tGFP mESC line differentiation, transfection and qPCR analysis. (B-C) *Ripk4* and *Grik1* overexpression were validated by qPCR. The data were normalized by *Gapdh* and control was set as 1.0. (D-G) The transcriptional expression levels of Hedgehog pathway genes: *Ptch1* (D), *Gli1* (E), *Gli2* (F), and *Gli3* (G) were examined by qPCR. Samples were normalized by *Gapdh* expression and the control was set as 1.0. For each condition, n = 4, paired t-test. Error bars indicate standard error of mean. Asterisks indicate statistical significance at P≤0.05.

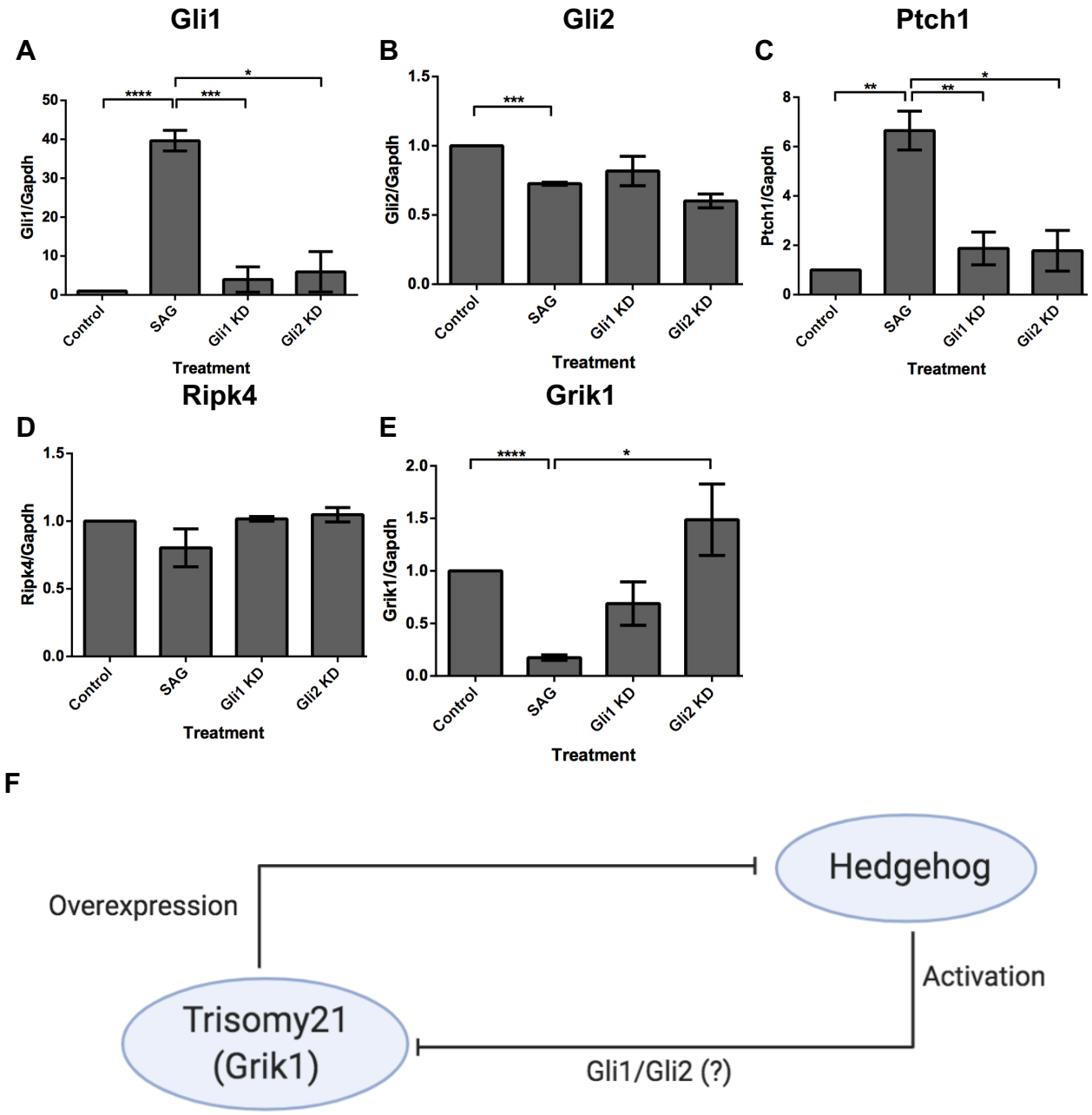

Figure 6: The activation of Hedgehog signaling induced suppressed *Grik1* expression and *Gli1/Gli2* knockdown recovered Grik1 expression level back.
(A-B) *Gli1* and *Gli2* knockdown were validated by qPCR. The data were normalized by *Gapdh* and control was set as 1.0. (C-E) The transcriptional expression levels of Hedgehog pathway genes: *Ptch1* (C), trisomy genes: *Ripk4* (D), and *Grik1* (E) were examined by qPCR. Samples were normalized by *Gapdh* expression and the control was set as 1.0. For each condition, n = 4, paired t-test. Error bars indicate standard error of mean. Asterisks indicate statistical significance, P≤0.05. (F) Proposed regulation mechanism between Trisomy 21(*Grik1*) and Hedgehog.

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